

Up-regulation of *miR-21* Mediates Resistance to Trastuzumab Therapy for Breast Cancer^{*[5]}

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Trastuzumab resistance emerges to be a major issue in anti-human epidermal growth factor receptor 2 (HER2) therapy for breast cancers. Here, we demonstrated that *miR-21* expression was up-regulated and its function was elevated in *HER2*⁺ BT474, SKBR3, and MDA-MB-453 breast cancer cells that are induced to acquire trastuzumab resistance by long-term exposure to the antibody, whereas protein expression of the *PTEN* gene, a *miR-21* target, was reduced. Blocking the action of *miR-21* with antisense oligonucleotides re-sensitized the resistant cells to the therapeutic activities of trastuzumab by inducing growth arrest, proliferation inhibition, and G₁-S cell cycle checking in the presence of the antibody. Ectopic expression of *miR-21* in *HER2*⁺ breast cancer cells confers resistance to trastuzumab. Rescuing *PTEN* expression with a p3XFLAG-*PTEN*-mut construct with deleted *miR-21* targeting sequence at its 3' UTR restored the growth inhibition of trastuzumab in the resistant cells by inducing *PTEN* activation and AKT inhibition. *In vivo*, administering *miR-21* antisense oligonucleotides restored trastuzumab sensitivity in the resistant breast cancer xenografts by inducing *PTEN* expression, whereas injection of *miR-21* mimics conferred trastuzumab resistant in the sensitive breast tumors via *PTEN* silence. Up-regulation of *miR-21* in tumor biopsies obtained from patients receiving pre-operative trastuzumab therapy was associated with poor trastuzumab response. Therefore, *miR-21* overexpression contributes to trastuzumab resistance in *HER2*⁺ breast cancers and antagonizing *miR-21* demonstrates therapeutic potential by sensitizing the malignancy to anti-HER2 treatment.

Overexpression of human epidermal growth factor receptor 2 (HER2/NEU/c-ERBB2),⁴ a member of the epidermal growth

factor receptor (EGFR) family of 185 kDa, is found in 20–35% of human breast cancers (1). Amplification of *HER2* is linked to aggressive tumor behavior and poor clinical outcome with shorter disease-free intervals and overall survival in patients with early and advanced breast cancers (2). Tremendous efforts have been made to develop HER2-targeting cancer therapies, and a most successful strategy is the recombinant humanized anti-HER2 monoclonal antibody trastuzumab (Herceptin) that specifically binds to the extracellular domain of HER2 and blocks its function. Clinical application of trastuzumab in adjuvant and metastatic settings has been shown to prolong the survival of patients with *HER2*⁺ breast cancers (3). However, the response rate to trastuzumab monotherapy is less than 35%, whereas ~60% of patients with *HER2*⁺ cancers on regimens combining trastuzumab with microtubule stabilizing drugs do not respond to treatment (4). Moreover, most patients who achieve an initial response develop resistance to trastuzumab within 1 year (5). Therefore, identifying the mechanisms responsible for trastuzumab resistance is important for the development of new therapeutic strategies.

A number of mechanisms have been suggested for trastuzumab resistance, including dysregulation of downstream signaling pathways and compensated signaling by other EGF family members or through alternative pathways (6). Among them, reduced *PTEN* expression is a strong indicator to predict trastuzumab resistance in breast cancer patients. Further mechanistic study showed that loss of *PTEN* function in *HER2*⁺ cancer cells leads to trastuzumab resistance by enhancing downstream PI3K/AKT phosphorylation and thus preventing trastuzumab-mediated growth arrest (7). However, how *PTEN* expression is silenced in trastuzumab resistance remains illusive.

Micro-RNAs (miRNAs) are a class of small non-coding RNAs of ~22 nucleotides in size that are endogenously expressed in mammalian cells. They regulate gene expression by repressing mRNA translation or cleaving target mRNA. As a new family of gene regulators, miRNAs are involved in modulating multiple cellular pathways, including cell proliferation, differentiation, and apoptosis, and thus may function as oncogenes or tumor suppressing genes (8). Among them, oncogenic miRNAs, including *miR-17-92* (9), *miR-19a* (10), *miR-21* (11), *miR-26a* (12), *miR-141* (13), *miR-216a* (14), *miR-217* (14), *miR-*

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⁴ The abbreviations used are: HER2/ERBB2, human epidermal growth factor receptor; *PTEN*, phosphatase and tensin homolog; ASO, antisense oligonu-

cleotide; miRNA, micro-RNA; PCNA, proliferating cell nuclear antigen; STAT3, signal transducer and activator of transcription 3; AP-1, activator protein 1; Foxo, forkhead family; CDC25A, cell division cycle 25 homolog A; PDCD4, programmed cell death protein 4; RHOB, *ras* homolog gene family, member B; qRT, quantitative reverse transcription.

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221 (15), miR-222 (16), and miR-494 (17), have been reported as direct regulators of tumor suppressing *PTEN* via targeting to its 3' UTR. By inhibiting the protein expression of PTEN, these miRNAs promote oncogenesis and progression of various carcinomas, and contribute to chemotherapeutic resistance. However, whether *PTEN*-targeting miRNAs are involved in trastuzumab resistance has not yet been clarified.

In the present study, we screened for miRNAs that were differentially expressed in the trastuzumab-resistant breast cancer cells, and identified that *miR-21* was up-regulated among the reported *PTEN*-targeting miRNAs. Antagonizing *miR-21* expression by specific antisense oligonucleotides (ASOs) increased PTEN expression, and restored sensitivity to trastuzumab *in vitro* and *in vivo*. Vice versa, ectopic expression of *miR-21* in *HER2*⁺ breast cancer cells reduced PTEN protein expression, and resulted in trastuzumab resistance *in vitro* and *in vivo*.

EXPERIMENTAL PROCEDURES

Patients and Tissue Samples—Primary ductal carcinomas of the breast with *HER2* gene amplification were obtained from 32 female breast cancer patients before and after pre-operative neoadjuvant Herceptin therapy in the breast tumor center, Sun-Yat-Sen Memorial Hospital, Sun-Yat-Sen University, from January 2008 to December 2009. All patients underwent pre-operative neoadjuvant therapy with 3–4 cycles of the tri-weekly TCH regimen (Taxotere, 75 mg/m², Carboplatin, AUC 6 mg/ml/min, and Herceptin, 8 mg/kg loading dose followed by 6 mg/kg every 3 weeks) according to NCCN guideline.

Breast tumor samples were obtained via core-needle biopsy prior to Herceptin therapy and from surgery after treatment. The collected tumor tissues were snap-frozen in liquid nitrogen for miRNA assay. In addition, the remaining tissues were embedded for studies of histology, immunohistochemistry, and *in situ* hybridization. All samples were collected with informed consent according to the internal review and ethics boards of the hospital.

In Situ Hybridization—*miR-21* expression was examined by *in situ* hybridization on the formalin-fixed and paraffin-embedded sections of breast cancers. Briefly, after dewaxing and rehydration, samples were digested with proteinase K, fixed again in 4% paraformaldehyde, hybridized with the 5' digoxin-labeled LNATM-modified *miR-21* probe (Exiqon) at 49.5 °C overnight, and then incubated overnight at 4 °C with anti-digoxin monoclonal antibody (Roche Applied Science). Followed by nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate staining solution in the dark, the sections were mounted and observed.

Immunohistochemistry—PTEN expression was examined by immunohistochemistry on paraffin-embedded tissue sections. Briefly, rabbit anti-PTEN polyclonal antibody (1:125, Cell Signaling Technology, Danvers, MA) was used as primary antibody for overnight incubation at 4 °C. The sections were subsequently treated with goat anti-rabbit secondary antibody, followed by further incubation with streptavidin-horseradish peroxidase complex. Diaminobenzidine (Dako, Carpinteria, CA) was used as a chromogen and sections were lightly counterstained with hematoxylin. The percentage of positively

staining tumor cells was calculated per field of view, with at least 20 view fields per section evaluated at ×400 magnification.

Cell Cultures and Treatment—SKBR3, MDA-MB-453, and BT474 cell lines were obtained from the American Type Culture Collection. Cells were maintained in RPMI 1640 with 10% fetal bovine serum. Trastuzumab (Herceptin) was obtained from Genentech and dissolved in sterile water. Stable cell lines were generated by transfection of p3XFLAG-*PTEN*-mut or p3XFLAG constructs followed by selection in puromycin. Transfection of the cells with miRNA mimics or miRNA ASOs (Genepharma) was performed using Lipofectamine (Invitrogen) as previously described (18).

miRNA Microarray Analysis—miRNA microarray analysis was done in the parental and resistant BT474 lines. Briefly, total RNA is harvested using TRIzol (Invitrogen) and the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. The samples are labeled using the miRCURYTM Hy3TM/Hy5TM Power labeling kit (Exiqon) and hybridized on the miRCURY LNA Array (Exiqon, version 11.0). Scanning was performed with the Axon GenePix 4000B microarray scanner. GenePix pro version 6.0 was used to read the raw intensity of the image. Unsupervised hierarchical clustering was performed on the miRNA expression profiling.

Real-time PCR Assay—cDNA was obtained by reverse transcription of total RNA using a TaqMan Reverse Transcription Kit (Applied Biosystems Inc.). Standard curves were generated and the relative amount of target miRNAs was normalized to U6 snRNA. Specially, the absolute copy number of mature miRNAs was determined by real-time quantitative reverse transcription PCR (qRT-PCR) using TaqMan Assays-on-Demand primer and probe sets along with TaqMan Universal PCR master mix (Applied Biosystems Inc.) for cDNA amplification. Amplification and analysis were performed on the ABI 7900 sequence detection system. Copies per cell were determined from total nanograms of RNA using an estimated 15 pg of total RNA per cell.

Northern Blot Analysis—An aliquot (10 μg) of total RNA was subjected to denaturing (7 mol/liter of urea) polyacrylamide (15% acrylamide) gel electrophoresis, transferred to Zetaprobe GT membrane (Bio-Rad), immobilized to the membrane by ultraviolet cross-linking, and hybridized overnight at 42 °C to ³²P-labeled deoxyoligonucleotide antisense to *miR-21*. The blots were reprobated with antisense oligo specific for U6 snRNA to show comparable RNA loading in each lane.

Luciferase Reporter Assay—To evaluate the function of *miR-21*, the 3' UTR of *PTEN* with a *miR-21* targeting sequence was cloned into a pMIR-REPORT luciferase reporter vector (Ambion). The assay was conducted as described (18). Briefly, the reporter vector plasmid was transfected using Lipofectamine 2000. To correct transfection efficiency, a luciferase reporter vector without the *miR-21* target was transfected in parallel. Luciferase activities in the cells were assayed using a luciferase assay kit (Promega, Madison, WI), and *miR-21* function was expressed as percentage of the luciferase activity of the reporter vector with *miR-21* target sequence over one without the *miR-21* target sequence.

Western Blotting—Protein extracts were resolved through 10% SDS-PAGE, transferred to nitrocellulose membranes,

probed with rabbit polyclonal antibodies against human PTEN (Upstate), human AKT or phosphor-AKT (Chemicon Int.), and then with peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Oncogene Research Product), and then visualized by chemiluminescence (Amersham Biosciences).

PTEN Phosphatase Assay—Cells treated with trastuzumab were incubated in lysis buffer (50 mM Tris-HCl, pH 7.6, 120 mM NaCl, 0.5% Nonidet P-40) containing protease inhibitor mixture (Roche Applied Sciences), and the cleared lysates were immunoprecipitated with anti-PTEN and protein G-agarose (Pierce). After overnight incubation at 4 °C and thorough washing in low-stringency buffer, containing 20 mM HEPES (pH 7.7), 50 mM NaCl, 0.1 mM EDTA, and 2.5 mM MgCl₂ and subsequently in phosphatase assay buffer lacking phosphatidylinositol 1,4,5-trisphosphate. PTEN phosphatase assays were performed for 40 min at 37 °C in 50 μl of buffer containing 100 mM Tris-HCl (pH 8.0), 10 mM DTT, and 100 μM water-soluble diC₈-phosphatidylinositol 1,4,5-trisphosphate substrate (Echelon, Salt Lake City, UT) in a 96-well plate. Release of phosphate from substrate was measured using Biomol Green Reagent (Biomol Research Lab, Inc., Plymouth Meeting, PA).

Measurements of Cell Viability, Proliferation, and Cell Cycle—Cell viability was measured using the [³H]thymidine incorporation assay. Briefly, [³H]thymidine (1 μCi) was added for 6 h to 2 × 10⁴ cells in octuplicate microtiter wells before harvesting and analysis by scintillation counting using a Top Count microplate reader (Packard). For colony-forming assay, cells were harvested and resuspended in medium supplemented with agar at a final concentration of 0.35%. Subsequently, 1 ml of the resulting cell suspension was plated in 12-well plates covered with medium supplemented with 0.6% agar, and cultured at 37 °C with 5% CO₂. The percentage of cells that formed into a spherical clone was calculated. For cell cycle analysis, cells were trypsinized, pelleted, and then resuspended in propidium iodide solution (Sigma), including 50 μg/ml of propidium iodide, 0.1 mg/ml of RNase A, and 0.05% Triton X-100, for a 40-min incubation at 37 °C and analyzed by flow cytometry using a FACScalibur instrument with CellQuest Software (BD Biosciences). Additionally, S phase cells were detected using 5-bromo-2'-deoxyuridine (BrdU) incorporation. Briefly, BrdU (Upstate) was added to the medium at 10 μM for 8- or 24-h incubations. BrdU incorporation in cancer cells was evaluated by immunostaining with a phycoerythrin-conjugated anti-BrdU antibody (Upstate, Temecula, CA) and analyzed by flow cytometry analysis.

Animal Experiment—Athymic nude female mice, 3–4 weeks old, were subcutaneously implanted with 1.5 mg of 60-day release 17β-estradiol pellets (Innovative Research, Sarasota, FL). Trastuzumab-resistant or parental BT474 cells (1 × 10⁷) in 100 μl of PBS per 100 μl of growth factor-reduced Matrigel (BD Biosciences) were injected into the mammary fat pads of the mice. When the xenografts reached ≈150 mm³, miR-21 ASO or control *Lin4* ASO (20 μg/injection, twice a week) was administered by multiple intratumor injection 72 h prior to intravenous injection of trastuzumab or control IgG (10 mg/kg, twice a week). Tumor volume was followed as volume = width² × length/2.

Statistics—The *in vitro* data were depicted as mean ± S.D. of three independent experiments performed in triplicate or in penultimate. Statistical analysis was performed by one-way analysis of variance and comparisons among groups were performed by independent sample *t* test or Bonferroni's multiple comparison *t* test. To measure the association between variables, Spearman order correlations were run.

RESULTS

Trastuzumab-resistant Breast Cancer Cells Overexpress miR-21—To obtain breast cancer cells with trastuzumab resistance, we cultured *HER2*⁺ cell lines, SKBR3, MDA-MB-453, and BT474, in the presence of low-dose trastuzumab (5 μg/ml) continuously for 6 months as reported elsewhere (19). The resulting cell lines grow at similar rates in the presence or absence of trastuzumab at 10 μg/ml for 3 days (data not shown). We next examined the micro-RNA expression profile in the parental and resistant BT474 lines. Using a cutoff of >2-fold changes in miRNA expression, we identified that a set of 14 miRNAs were reduced and another set of seven miRNAs were up-regulated in the resistant line as compared with the parental one (supplemental Table S1). Among the reported *PTEN*-targeting miRNAs, only *miR-21* and *miR-141* were up-regulated in trastuzumab-resistant BT474 cells (supplemental Table S1). However, the levels of *miR-216a*, *miR-217*, *miR-221*, and *miR-222* were comparable in the parental and resistant lines, and the rest of the *PTEN*-targeting miRNAs, including *miR-17-92*, *miR-19a*, *miR-26a*, and *miR-494*, were not detected in both lines (data not shown).

To quantify the detectable *PTEN*-targeting miRNAs in both BT474 lines, we performed qRT-PCR using specific primers against these miRNAs. In agreement with the microarray data, *miR-21* and *miR-141* in trastuzumab-resistant BT474 cells were ~8.17- (*p* < 0.001) and 1.9-fold higher (*p* < 0.05), respectively, than the parental ones, whereas the expression of *miR-216a*, *miR-217*, *miR-221*, and *miR-222* was not different in both lines (*p* > 0.05, Fig. 1A). Similarly, *miR-21* was also significantly elevated in trastuzumab-resistant SKBR3 (≈5.8-fold versus parental line, *p* < 0.001) and MDA-MB-453 (≈3.3-fold versus parental line, *p* < 0.01) lines as compared with the corresponding trastuzumab-sensitive parental lines (Fig. 1B). Furthermore, we calculated the absolute quantification of *miR-21* based on standard curves of a serial dilution of the *miR-21* synthetic miRNA. The copy numbers of miRNA per cell were 1.16 × 10³, 0.98 × 10³, and 1.58 × 10³ in the parental BT474, SKBR3, and MDA-MB-453 cells, respectively, but increased to 1.39 × 10⁴ (≈12.0-fold), 0.96 × 10⁴ (≈9.7-fold), and 2.03 × 10⁴ (≈12.8-fold) in the corresponding trastuzumab-resistant cells (*p* < 0.001, supplemental Fig. S1A). Moreover, up-regulation of *miR-21* was also confirmed in resistant BT474, SKBR3, and MDA-MB-453 by Northern blot analysis (Fig. 1C). Transfecting the trastuzumab-resistant lines with *miR-21* ASO, instead of a nonspecific *Lin4* ASO or mock transfection, reduced the miRNA expression to the level comparable with the parental lines (*p* < 0.001, Fig. 1D). Vice versa, transfection with a *miR-21* mimic up-regulated the miRNA expression by 5–8-fold, respectively, in the *HER2*⁺ parental lines (*p* < 0.001, Fig. 1D). Next, we monitored the dynamics of *miR-21* expression in

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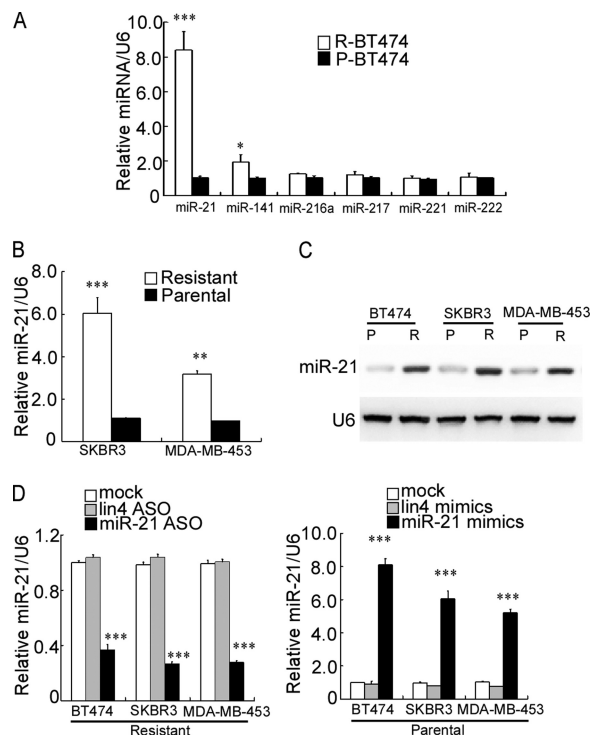


FIGURE 1. *miR-21* expression is up-regulated in trastuzumab-resistant *HER2*⁺ breast cancer cells. *A*, real-time qRT-PCR demonstrated the expression of a panel of *PTEN*-targeting miRNAs in the trastuzumab-resistant *HER2*⁺ BT474 breast cancer cells, which was normalized to the parental line. Trastuzumab-resistant lines were obtained by continuously culturing the parental lines in 5 $\mu\text{g}/\text{ml}$ of trastuzumab for 6 months. *, $p < 0.05$, and ***, $p < 0.001$ as compared with the parental lines. *B*, qRT-PCR for *miR-21* expression in the trastuzumab-resistant *HER2*⁺ SKBR3 and MDA-MB-453 lines, which was normalized to their parental lines. **, $p < 0.01$, and ***, $p < 0.001$ as compared with the parental lines. *C*, Northern blot analysis for *miR-21* expression in the trastuzumab-resistant and parental lines. *D*, qRT-PCR for *miR-21* expression in the trastuzumab-resistant BT474, SKBR3, and MDA-MB-453 lines mock transfected or transfected with a *Lin4* ASO or a *miR-21* ASO (left) and in the trastuzumab-sensitive parental BT474, SKBR3, and MDA-MB-453 lines mock transfected or transfected with a *Lin4* mimic or a *miR-21* mimic (right). The data were normalized to mock transfected cells. ***, $p < 0.001$ as compared with mock transfected cells.

BT474 cells treated continuously with 5 $\mu\text{g}/\text{ml}$ of trastuzumab for various periods of time, ranging from 1 to 8 months. Determined by qRT-PCR, *miR-21* expression increased as treatment prolonged and reached a plateau at 6 months (supplemental Fig. S1B), which was reminiscent of the changes in IC_{50} concentration of trastuzumab in the BT474 lines (supplemental Table S2). These data suggest that *miR-21* up-regulation is induced in *HER2*⁺ breast cancer cells in the process of acquired trastuzumab resistance.

***miR-21* Up-regulation in Trastuzumab-resistant Cells Leads to *PTEN* Reduction**—To investigate *miR-21* function, we transfected a luciferase reporter containing the 3' UTR of *PTEN* with a *miR-21* seed site into the trastuzumab-resistant breast cancer cells as well as their corresponding parental lines (Fig. 2A). In agreement with *miR-21* overexpression, luciferase activities were suppressed by $74.7 \pm 1.10\%$ ($p < 0.001$), $58.42 \pm 3.42\%$ ($p < 0.01$), and $82.30 \pm 2.37\%$ ($p < 0.001$) in trastuzumab-resistant BT474, SKBR3, and MDA-MB-453 breast cancer cells, respectively, whereas suppressions in their parental lines were only 6.2–10.7% (Fig. 2B). However, cotransfection with *miR-21* ASOs significantly relieved the suppression in luciferase activ-

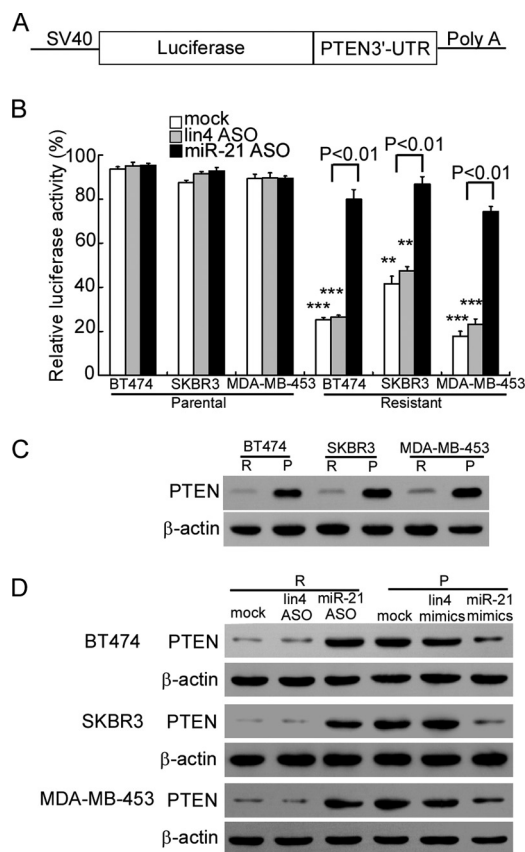


FIGURE 2. Up-regulated *miR-21* in trastuzumab-resistant cells reduces *PTEN* expression. *A*, diagram of a pMIR-REPORT luciferase reporter vector containing *PTEN* 3' UTR with a *miR-21* seed site (pMIR-Luc-*PTEN*3'UTR). *B*, luciferase activities in the trastuzumab-resistant and the parental BT474, SKBR3, or MDA-MB-453 cells transfected with a pMIR-Luc-*PTEN*3'UTR in parallel to transfection with a luciferase reporter vector without *miR-21* target (pMIR-Luc- Δ *PTEN*3'UTR). The cells were mock co-transfected or co-transfected with *Lin4*-ASO or *miR-21* ASO as indicated. Data were expressed as relative luciferase activity, the percentage of luciferase activity in the cells transfected with pMIR-Luc-*PTEN*3'UTR over those with pMIR-Luc- Δ *PTEN*3'UTR. **, $p < 0.01$, and ***, $p < 0.001$ versus mock transfected parental lines. *C* and *D*, Western blot analysis for *PTEN* expression in parental (*P*) or trastuzumab-resistant (*R*) BT474, SKBR3, or MDA-MB-453 cells that were untransfected (*C*) or mock transfected or transfected with *Lin4* ASO or *miR-21* ASO (*D*). β -Actin was used as a loading control.

ity in the trastuzumab-resistant lines ($p < 0.01$ versus *Lin4* ASO, Fig. 2B), but did not significantly influence that in the parental cells ($p > 0.05$ versus *Lin4* ASO, Fig. 2B). Therefore, *miR-21* overexpression in the trastuzumab-resistant breast cancer cells is accompanied by elevated targeting function of the miRNA.

PTEN activation is involved in the anti-tumor mechanism of trastuzumab and its deficiency in tumor cells predicts primary resistance to trastuzumab (7). Herein, we examined *PTEN* expression in breast tumor lines with acquired trastuzumab resistance. Although *PTEN* mRNA remained unchanged in trastuzumab-resistant BT474, SKBR3, and MDA-MB-453 cells as compared with their parental lines (supplemental Fig. S2), its protein levels were significantly reduced (Fig. 2C). Furthermore, transfecting the trastuzumab-resistant breast cancer lines with a *miR-21* ASO substantially increased *PTEN* protein, whereas *miR-21* mimics significantly reduced the protein expression of *PTEN* in the parental lines (Fig.

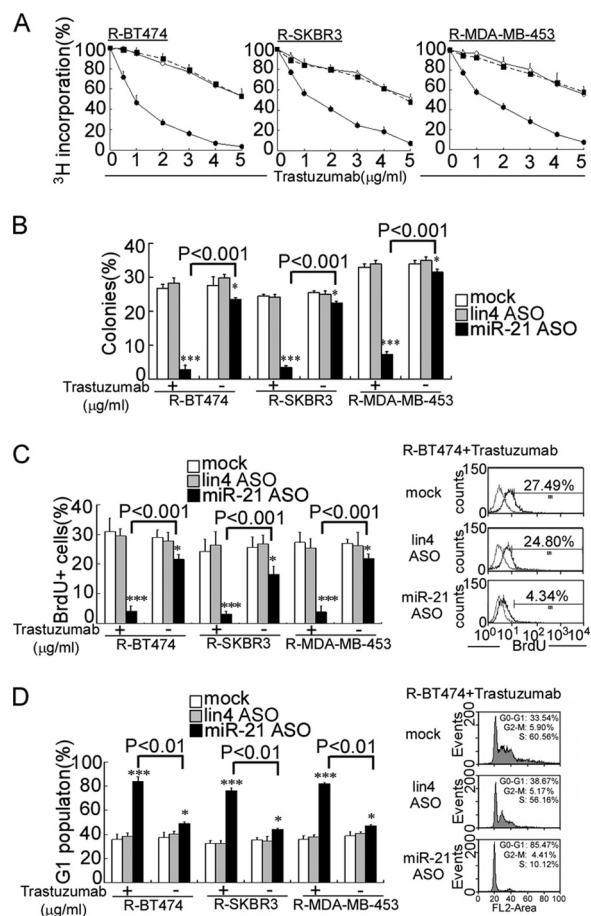


FIGURE 3. *miR-21* ASO increases trastuzumab sensitivity in the resistant breast cancer cells. A, [^3H]thymidine incorporation assays for the trastuzumab-resistant BT474, SKBR3, or MDA-MB-453 cells that were mock transfected (■) or transfected with *Lin4* ASO (◇) or *miR-21* ASO (●) and were treated with increasing concentrations of trastuzumab. B, percentage of the anchorage-independent colony formation determined by soft agar assays in the trastuzumab-resistant BT474, SKBR3, or MDA-MB-453 cells treated as in A. *, $p < 0.05$, and ***, $p < 0.001$ versus mock transfected cells. C, percentage of BrdU $^+$ cells determined by flow cytometric analysis of BrdU immunostaining (left) and representative flow cytometric histograms of BrdU immunostaining (right) in the trastuzumab-resistant BT474, SKBR3, or MDA-MB-453 cells treated as in A. *, $p < 0.05$, and ***, $p < 0.001$ versus mock transfected cells. D, percentage of the cells in the G_1 phase determined by flow cytometric analysis for cell cycles (left) and representative flow cytometric histograms of cell cycle analysis in the trastuzumab-resistant BT474, SKBR3, or MDA-MB-453 cells treated as in A. *, $p < 0.05$; and ***, $p < 0.001$ versus mock transfected cells.

2D). Therefore, PTEN reduction in *HER2* $^+$ tumor cells with acquired trastuzumab resistance was probably induced by *miR-21* up-regulation.

Blockade of *miR-21* Retrieves Trastuzumab Sensitivity—To investigate whether *miR-21* overexpression may contribute to trastuzumab resistance in *HER2* $^+$ breast cancer cells, we inhibited *miR-21* expression with ASOs. The tumor cells were treated with trastuzumab at increasing concentrations for 3 days, and growth inhibition induced by the drug was measured by [^3H]thymidine incorporation assay. Transfecting the resistant BT474, SKBR3, and MDA-MB-453 lines with *miR-21* ASOs, but not *Lin4* ASOs or mock transfection, markedly sensitized the tumor cells to trastuzumab, as the IC_{50} decreased by roughly 3.5–5.3-fold, respectively (Fig. 3A). However, without trastuzumab treatment, transfection of *miR-21* ASOs alone

only slightly reduced the [^3H]thymidine incorporation in the resistant lines as compared with mock transfection ($p < 0.05$, supplemental Fig. S3). We next tested whether *miR-21* ASOs may inhibit anchorage-independent growth of the resistant breast tumor cells in the presence of trastuzumab. The cells were treated with 10 $\mu\text{g}/\text{ml}$ of trastuzumab for 3 days. As shown by the quantifications of soft agar assay, transfection with *miR-21* ASO resulted in 9.5- ($p < 0.001$), 7.0- ($p < 0.001$), and 4.5- ($p < 0.001$)-fold reduction in the number of colonies formed by the resistant BT474, SKBR3, and MDA-MB-453 lines, respectively (Fig. 3B). However, without trastuzumab, *miR-21* ASOs alone reduced colony formation only by $\sim 3.58 \pm 1.72\%$ ($p < 0.05$, Fig. 3B). Therefore, blocking *miR-21* with ASOs enhances trastuzumab sensitivity in the *HER2* overexpressing breast cancer cells with acquired resistance to the drug.

We next asked whether *miR-21* inhibition may reverse the refractoriness of the tumor cells to the anti-proliferative effect of trastuzumab. Under treatment with trastuzumab, the proliferation of breast tumor cells was measured by their ability to incorporate BrdU, and cell cycle profiles were examined by flow cytometric analysis for propidium iodide staining. In the presence of trastuzumab, the resistant BT474, SKBR3, and MDA-MB-453 breast cancer cells transfected with *miR-21* ASO, instead of *Lin4* ASO or mock transfection, showed a substantial ($\approx 24\%$) reduction in BrdU staining ($p < 0.001$, Fig. 3C). However, *miR-21* ASO alone without trastuzumab results in a mild decrease in BrdU incorporation in the resistant lines ($p < 0.05$, Fig. 3C). Consistently, transfecting the resistant lines with *miR-21* ASO markedly increased the percentage of G_1 population in the presence of trastuzumab ($p < 0.001$), but slightly affected the magnitude of G_1 -S arrest without trastuzumab ($p < 0.05$, Fig. 3D). These data suggested that *miR-21* suppression resensitizes the resistant breast cancer cells to the anti-proliferative and cell-cycle blocking effect of trastuzumab.

Ectopic *miR-21* Expression Confers Trastuzumab Resistance on Breast Cancer Cells—Because our data have shown that *miR-21* inhibition in trastuzumab-resistant breast cancer lines retrieves their sensitivity, we next investigated whether enforced *miR-21* expression in the sensitive *HER2*-overexpressing cancer cells may lead to trastuzumab resistance. Contrary to *miR-21* suppression in trastuzumab-resistant cells with *HER2* overexpression, transfecting the parental BT474, SKBR3, or MDA-MB-453 cells with *miR-21* mimics, but not with an irrelevant *Lin4* mimics or mock transfection, significantly reduced their sensitivity to the growth inhibitory effect of trastuzumab, as the IC_{50} of trastuzumab in these cells as determined by the [^3H]thymidine incorporation assay was 4.5–6.8-fold higher than the mock transfected cells, respectively ($p < 0.01$, Fig. 4A). Additionally, when the trastuzumab-sensitive cells were treated with 10 $\mu\text{g}/\text{ml}$ of trastuzumab for 3 days, soft agar assay demonstrated that colony formation in the cells transfected with *miR-21* mimics was 1.8–2.0-fold higher than the mock transfected cells ($p < 0.001$, Fig. 4B). Moreover, trastuzumab treatment did not result in any changes of colony formation in the cells with enforced *miR-21* expression ($p > 0.05$). However, in the absence of trastuzumab, *miR-21* mimics increased [^3H]thymidine incorporation by nearly 7% ($p < 0.05$)

miR-21 Mediates Trastuzumab Resistance

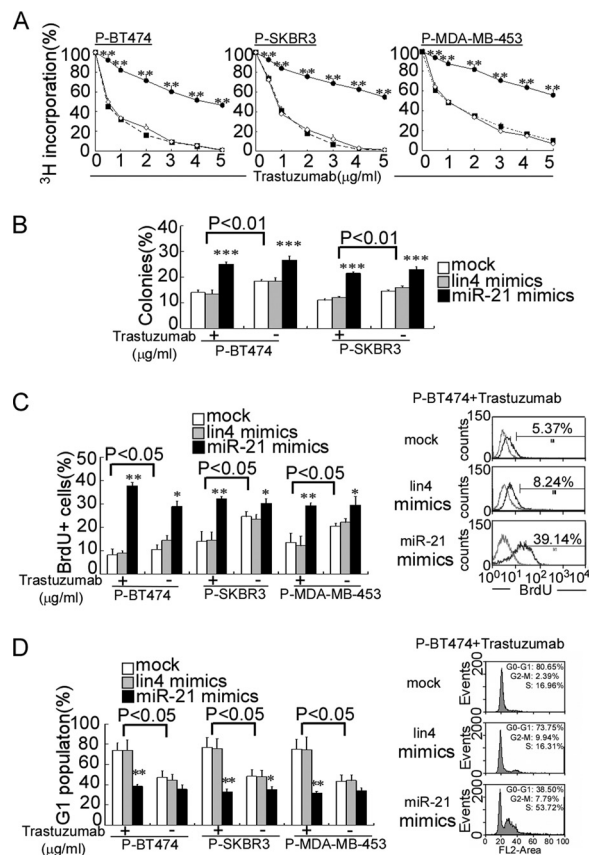


FIGURE 4. Ectopic *miR-21* expression confers trastuzumab resistance in breast cancer cells. *A*, [^3H]thymidine incorporation assays for the parental BT474, SKBR3, or MDA-MB-453 cells mock transfected (■) or transfected with *Lin4* (◇) or *miR-21* mimics (●) and treated with increasing concentrations of trastuzumab. **, $p < 0.01$ versus mock transfected cells. *B*, percentage of the anchorage-independent colony formation determined by soft agar assays in the parental BT474 and SKBR3 cells treated as in *A*, parental MDA-MB-453 cells did not form colonies. ***, $p < 0.001$ versus mock transfected cells. *C*, percentage of BrdU⁺ cells determined by flow cytometric analysis of BrdU immunostaining (left) and representative flow cytometric histograms of BrdU immunostaining (right) in the parental BT474, SKBR3, and MDA-MB-453 cells treated as in *A*. *, $p < 0.05$; and **, $p < 0.01$ versus mock transfected cells; *D*, percentage of cells in the G₁ phase determined by flow cytometric analysis for cell cycles (left) and representative flow cytometric histograms of cell cycle analysis in the parental BT474, SKBR3, or MDA-MB-453 cells treated as in *A*. *, $p < 0.05$; and **, $p < 0.01$ versus mock transfected cells.

and colony formation by ~8% ($p < 0.01$) in the parental lines (supplemental Fig. S4 and Fig. 4B). These data provided evidence that ectopic *miR-21* expression confers trastuzumab resistance on *HER2*⁺ breast cancer cells.

Furthermore, BrdU incorporation in the presence of 10 $\mu\text{g/ml}$ of trastuzumab for 3 days remained unchanged in the parental BT474, SKBR3, or MDA-MB-453 lines that were transfected with *miR-21* mimics ($p > 0.05$, Fig. 4C), but was substantially reduced in control cells that were mock transfected or transfected with *Lin4* mimics ($p < 0.01$, Fig. 4C). In agreement, under treatment with trastuzumab, the parental lines transfected with *miR-21* mimics did not exhibit any significant enrichment of the G₁ population ($p > 0.05$) determined by propidium iodide staining followed by flow cytometric analysis, whereas a notable increase in the number of mock-transfected cells in G₁ was observed ($p < 0.01$, Fig. 4D). Taken together, these results indicate that ectopic *miR-21* expression overcomes the G₁-S arrest and proliferation inhibition medi-

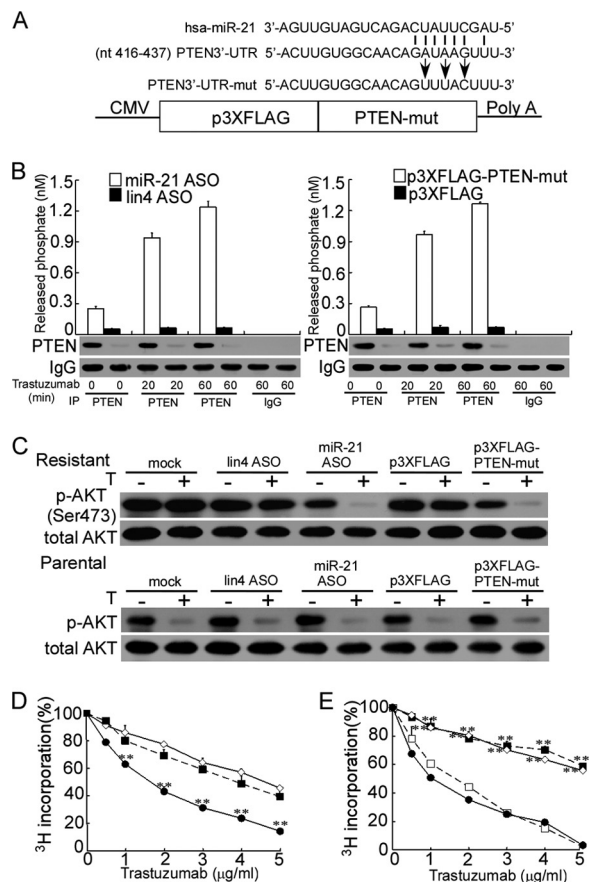


FIGURE 5. Retrieving PTEN expression re-sensitizes breast cancer cells to trastuzumab. *A*, diagram of the p3XFLAG-PTEN-mut construct with three mutated nucleotides (arrowheads) at the *miR-21* binding site within the 3' UTR of PTEN. *B*, PTEN phosphatase activities (upper) and Western blotting for PTEN expression (lower) in the membrane precipitates with an anti-PTEN antibody or a control isotype IgG in the trastuzumab-resistant BT474 cells that were transfected with *miR-21* ASO, *Lin4* ASO (left), or with p3XFLAG-PTEN-MUT or empty p3XFLAG vector (right) and treated with trastuzumab for the indicated periods of time. *C*, Western blotting for the expression of the total protein and the phosphorylated protein of AKT at Ser-473 in the trastuzumab-resistant and -sensitive BT474 cells mock transfected or transfected with *miR-21* ASO, *Lin4* ASO, p3XFLAG-PTEN-mut, or p3XFLAG vector alone. *D*, [^3H]thymidine incorporation assays for the trastuzumab-resistant BT474 cells that were mock transfected (■) or transfected with p3XFLAG-PTEN-mut (●) or p3XFLAG vector alone (◇) and treated with increasing concentrations of trastuzumab; **, $p < 0.01$ versus cells transfected with an empty p3XFLAG vector. *E*, [^3H]thymidine incorporation assays for the parental BT474 cells mock transfected (□) or transfected with *miR-21* mimics (■) or co-transfected with *miR-21* mimics and p3XFLAG-PTEN-mut (●) or p3XFLAG (◇) and treated with increasing concentrations of trastuzumab. **, $p < 0.01$ versus mock transfected cells.

ated by trastuzumab treatment, and thus induces drug resistance.

***miR-21* Induces Trastuzumab Resistance via PTEN Inhibition**—To investigate whether *miR-21*-induced PTEN reduction is responsible for trastuzumab resistance in breast cancer cells, we retrieved PTEN expression in the resistant BT474 line using a *miR-21* ASO or a p3XFLAG-PTEN-mut construct with three nucleotides mutated in the reported *miR-21* seed sequence (20) induced by PCR site-directed mutagenesis (Fig. 5A). Transfection of the trastuzumab-resistant BT474 cells with p3XFLAG-PTEN-mut, instead of an empty p3XFLAG vector, restored PTEN expression to the level comparable with the parental line (supplemental Fig. S5),

which was in line with transfection with *miR-21* ASO as shown above.

It has been shown that trastuzumab exerts its anti-tumor effect by enhancing PTEN phosphatase activity and inhibiting AKT phosphorylation in *HER2*⁺ breast cancer cells (7). Therefore, we evaluated whether retrieving PTEN expression in the trastuzumab-resistant cancer cells may recover their capability of PTEN activation upon treatment with the antibody. Interestingly, when PTEN expression was restored in the resistant BT474 cells with *miR-21* ASO or p3XFLAG-*PTEN*-mut, treatment with trastuzumab for 1 h dramatically increased PTEN phosphatase activities, measured following immunoprecipitation of a similar amount of PTEN protein using an anti-PTEN antibody (Fig. 5B), and the activity remained stably high when treated for 24 or 72 h (data not shown). Aligned with restoration of PTEN expression and activity, the resistant cells transfected with *miR-21* ASO or p3XFLAG-*PTEN*-mut, but not with *Lin4* ASO or p3XFLAG vector, were re-sensitized to the inhibitory effect of trastuzumab on AKT phosphorylation (Fig. 5C), similar to their parental lines. However, trastuzumab-mediated AKT inhibition in the parental lines was independent of transfection with *miR-21* ASO or p3XFLAG-*PTEN*-mut (Fig. 5C). These data suggest that retrieving PTEN expression in the resistant breast cancer cells restores trastuzumab-mediated AKT phosphorinhibition.

Furthermore, we evaluated whether restoring PTEN expression and activation may re-sensitize the resistant cancer cells to trastuzumab treatment in the context of *miR-21* overexpression. Transfecting the resistant BT474 cells with p3XFLAG-*PTEN*-mut restored the inhibitory effect of trastuzumab on [³H]thymidine incorporation, as the IC₅₀ decreased by ~2.6-fold as compared with cells transfected with an empty p3XFLAG vector ($p < 0.01$, Fig. 5D). On the other hand, co-transfecting the parental BT474 cells with *miR-21* mimics and p3XFLAG-*PTEN*-mut did not change [³H]thymidine incorporation significantly upon treatment with various concentrations of trastuzumab ($p > 0.05$, Fig. 5E), whereas co-transfection of *miR-21* mimics and the empty p3XFLAG vector significantly increased the IC₅₀ concentration of trastuzumab ($p < 0.01$, Fig. 5E). Therefore, retrieving PTEN expression may abolish trastuzumab resistance induced by *miR-21* overexpression.

miR-21-mediated PTEN Silence Confers Trastuzumab Resistance on Breast Tumor Xenografts—To investigate whether *miR-21* confers trastuzumab resistance in breast cancers *in vivo*, we treated the athymic nude mice inoculated with BT474 cells in their mammary fat pads with trastuzumab for 3 weeks and followed the growth of tumors. This regimen of trastuzumab did not influence *miR-21* expression in the xenografts, as the *miR-21* level in the xenografts determined by qRT-PCR was comparable in trastuzumab- and IgG-treated mice ($p > 0.05$, supplemental Fig. S6), which was not significantly different from that of the xenografts harvested before trastuzumab treatment ($p > 0.05$, supplemental Fig. S6).

When the xenografts of the trastuzumab-resistant BT474 line reached $\approx 150 \text{ mm}^3$, *miR-21* ASO (20 μg /injection, twice a week) was injected into the tumors 72 h prior to intravenous injection of trastuzumab (10 mg/kg, twice a week). Without

trastuzumab treatment, injection with *miR-21* ASO only minimally inhibited tumor growth as compared with non-relevant *Lin4* ASO ($p < 0.05$, Fig. 6A and supplemental Fig. S7A). Although trastuzumab did not influence the growth rate of the resistant BT474 xenografts, following intratumor injection with *miR-21* ASO, the magnitude of tumor inhibition was significantly more profound in animals receiving trastuzumab dosing than those without treatment with the antibody ($p < 0.01$, Fig. 6A and supplemental Fig. S7A), suggesting that *miR-21* inhibition may sensitize trastuzumab-resistant breast cancers to the antibody. On the other hand, treatment with trastuzumab inhibited the growth of tumor xenografts in nude mice inoculated with the parental BT474 line ($p < 0.01$, Fig. 6B and supplemental Fig. S7B), but was unable to retard tumor growth when *miR-21* mimics (15 μg /injection, twice a week) were injected into the xenografts 72 h prior to trastuzumab treatment ($p > 0.05$, Fig. 6B). However, without trastuzumab treatment, *miR-21* mimics alone revealed a trend to accelerate the growth rate of parental BT474 xenografts, but did not reach statistical significance ($p > 0.05$, Fig. 6B).

In agreement with the changes in tumor growth rate, trastuzumab treatment reduced the percentage of PCNA⁺ cancer cells in the trastuzumab-resistant BT474 xenografts pretreated with *miR-21* ASO ($p < 0.01$, Fig. 6C and supplemental Fig. S7C), but failed to do so in those pretreated with *Lin4* ASO ($p > 0.05$). Vice versa, trastuzumab was unable to reduce PCNA⁺ cancer cells in the parental BT474 xenografts pretreated with *miR-21* mimics ($p > 0.05$), whereas it significantly reduced the one in those pretreated with *Lin4* mimics ($p < 0.01$, Fig. 6D and supplemental Fig. S7D). However, intratumor injection with *miR-21* ASO in trastuzumab-resistant BT474 xenografts or with *miR-21* mimics in the parental BT474 xenografts without trastuzumab treatment only slightly changed PCNA expression in breast cancer cells ($p < 0.05$, Fig. 6, C and D, and supplemental Fig. S7, C and D). These data suggest that *miR-21* overexpression in breast cancer cells enhances their resistance to the proliferation inhibitory effect induced by trastuzumab.

To further evaluate whether *in vivo* trastuzumab resistance in breast cancers mediated by *miR-21* overexpression is related to *PTEN* gene silence, we examined PTEN expression in the xenografts using immunohistochemistry. As shown in Fig. 6D, PTEN expression in the tumor xenografts of the parental BT474 line was effectively inhibited ($p < 0.01$, supplemental Fig. S7F), by biweekly intratumor injection of *miR-21* mimics for 3 consecutive weeks when ErbB2 expression remained the same (not shown). Vice versa, repetitive *miR-21* ASO injection into the xenografts of the trastuzumab-resistant BT474 line resulted in increased PTEN, but not ErbB2 (not shown), expression in the xenografted cancer cells ($p < 0.01$, Fig. 6C and supplemental Fig. S7E). Therefore, *miR-21* overexpression in breast tumor xenografts results in trastuzumab resistance probably by inhibiting PTEN expression.

Overexpression of miR-21 Is Associated with Trastuzumab Resistance in Breast Cancer Patients—To further evaluate the clinical significance of *miR-21* overexpression in trastuzumab resistance, we examined the expression of *miR-21* in primary breast cancers from 32 patients receiving neoadjuvant trastuzumab therapy and correlated it with tumor response to the

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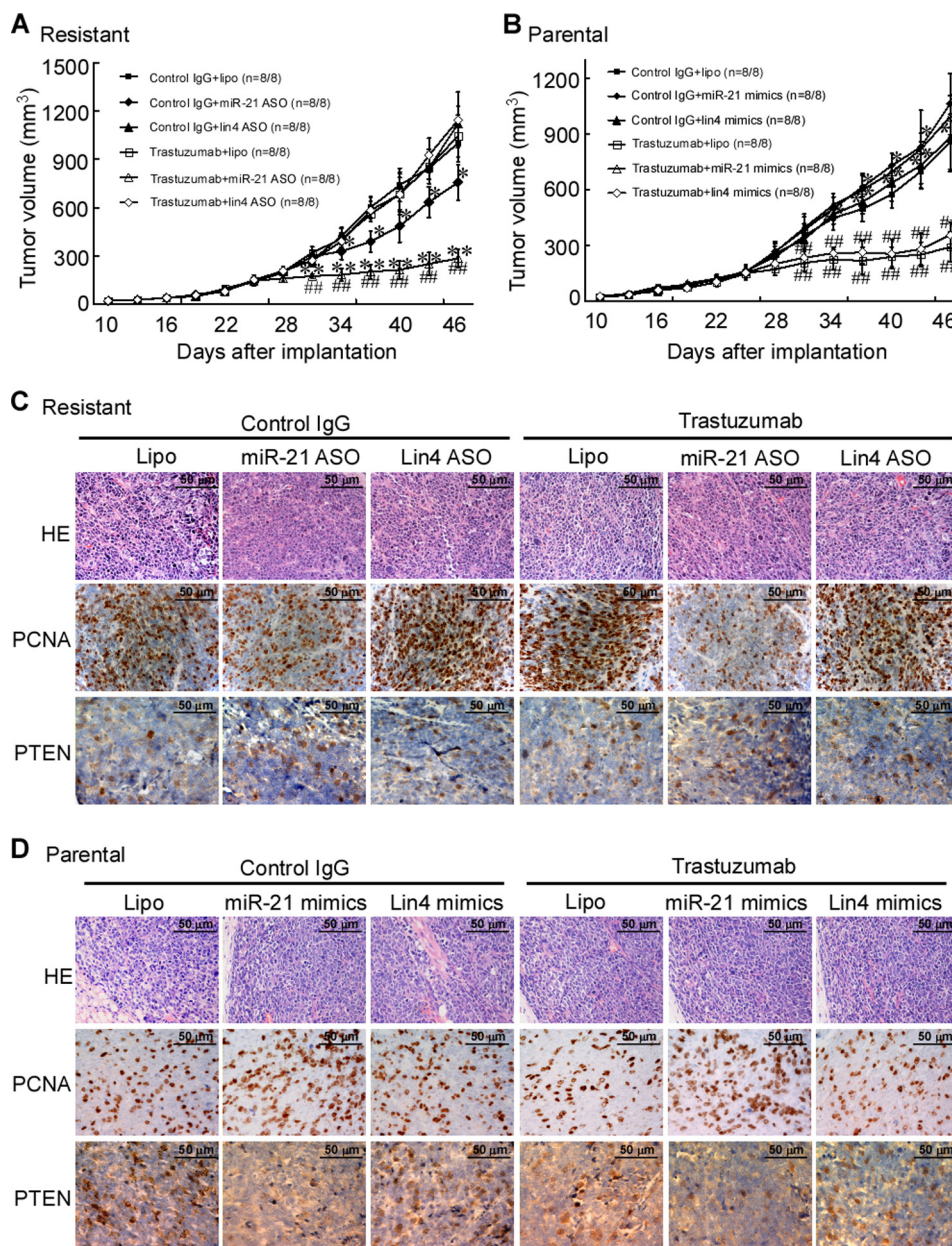


FIGURE 6. miR-21 confers trastuzumab resistance on HER2⁺ breast cancer xenografts in vivo. A and B, tumor volume was measured in athymic nude mice xenografted in the mammary fat pads with trastuzumab-resistant (A) or parental (B) BT474 (1×10^7 cells) breast cancer cells. When the xenografts were palpable (around 150 mm³ in diameter), intratumor injection with Lipofectamine (5 μ l) alone or in complex with 20 μ g of miR-21 ASO or Lin4 ASO for the resistant BT474 xenografts (A), or with 15 μ g of miR-21 or Lin4 mimics for the parental BT474 xenografts (B), followed by intravenous injection of trastuzumab or an isotype IgG 72 h later at 10 mg/kg, was performed biweekly for 3 consecutive weeks. *, $p < 0.05$; and **, $p < 0.01$ as compared with intratumor injection with Lipofectamine alone. ##, $p < 0.01$ versus xenografts without trastuzumab treatment. C and D, microscopic images of H&E staining and immunohistochemical staining for HER2, PCNA, and PTEN in the trastuzumab-resistant BT474 xenografts (C) treated as in A, and the parental BT474 xenografts (D) treated as in B.

drug, determined by the Response Evaluation Criteria in Solid Tumors (RECIST) (supplemental Table S3). *In situ* hybridization for miR-21 demonstrated that expression of the miRNA in tumor biopsies obtained prior to trastuzumab therapy was more abundant in the resistant tumors than the sensitive ones (Fig. 7, A and B). This was further confirmed by qRT-PCR showing that miR-21 was ~2.7-fold higher in the trastuzumab-resistant tumors than the sensitive ones ($p < 0.01$, Fig. 7C). Moreover, miR-21 expression was further up-regulated following trastuzumab therapy in both the resistant and sensitive tumors, but the difference did not

reach statistical significance (Fig. 7, B and C), implying that given a prolonged period of trastuzumab treatment, acquired trastuzumab resistance due to induction of miR-21 may occur. In line with miR-21 up-regulation, the protein expression of PTEN, a miR-21 target, was significantly lower in trastuzumab-resistant tumors as compared with the sensitive ones, determined by immunohistochemistry before and after trastuzumab treatment (Fig. 7, D and E). Moreover, miR-21 expression in breast cancer cells was reversely correlated with PTEN expression (Fig. 7F), and the correlation was higher in tumors after trastuzumab therapy (Fig. 7F, $p <$

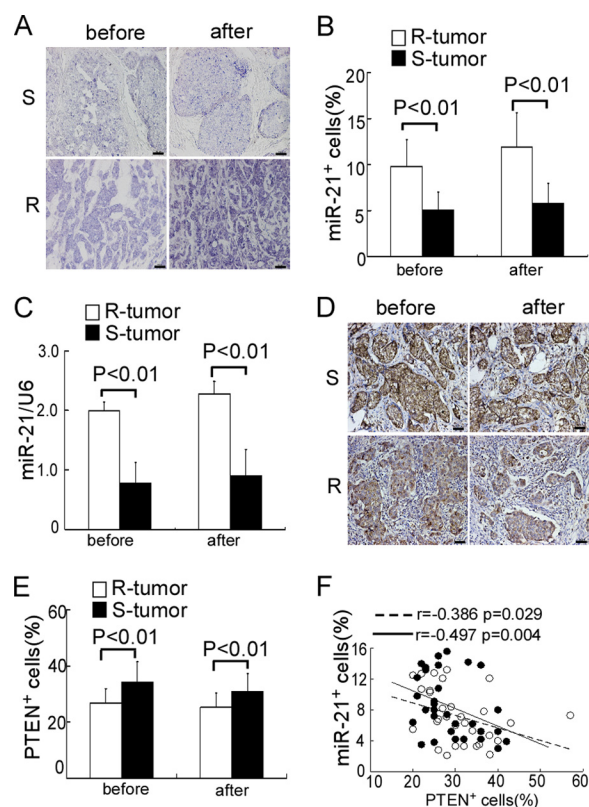


FIGURE 7. Overexpression of *miR-21* is associated with trastuzumab resistance in breast cancer patients. *A*, microscopic images ($\times 200$) of *in situ* hybridization for *miR-21* in trastuzumab-resistant (*R*) and -sensitive tumors (*S*) before and after neoadjuvant trastuzumab therapy. *B*, percentage of *miR-21* positive (*miR-21*⁺) cells in the trastuzumab-resistant and -sensitive tumors before and after neoadjuvant trastuzumab therapy. $p < 0.01$ R-tumors versus S-tumors. *C*, quantitative RT-PCR for *miR-21* expression in trastuzumab-resistant and -sensitive tumors before and after neoadjuvant trastuzumab therapy. $p < 0.01$ R-tumors versus S-tumors. *D*, microscopic images ($\times 200$) of immunohistochemical staining for PTEN expression in the trastuzumab-resistant and -sensitive tumors before and after trastuzumab therapy. *E*, percentage of PTEN positive (*PTEN*⁺) cells in trastuzumab-resistant and -sensitive tumors before and after neoadjuvant trastuzumab therapy. $p < 0.01$ R-tumors versus S-tumors. *F*, expression of *miR-21* and PTEN in each individual case of 32 breast cancer patients before (\circ) and after (\bullet) neoadjuvant trastuzumab therapy demonstrated in scatter diagrams. Correlation coefficients were demonstrated and $p < 0.01$ R-tumors versus S-tumors.

0.01) than before (Fig. 7*F*, $p < 0.05$). These data suggest that *miR-21* up-regulation is associated with PTEN reduction and trastuzumab resistance in breast cancers.

DISCUSSION

Trastuzumab is a model of highly specific targeted cancer therapy for *HER2* overexpressing breast cancers, but the tumors may be initially resistant to the antibody or acquire the trait of resistance in the course of treatment (6). In the present study, we demonstrate that *miR-21* up-regulation is induced in the process of acquired trastuzumab resistance in *HER2* overexpressing breast cancer cells, and contributes to resistance by inhibiting PTEN expression. Retrieving PTEN expression in trastuzumab-resistant cells restores their sensitivity to the antibody, whereas ectopic expression of *miR-21* in the previously sensitive cells confers trastuzumab resistance via PTEN inhibition. Clinically, up-regulation of *miR-21*, accompanied by reduced PTEN, is associated with poor trastuzumab response in breast cancers.

Altered expression and dysfunction of miRNAs have been well documented in nearly all types of human malignancies, and numerous miRNAs are involved in tumor formation and progression by regulating the expression and action of many oncogenes and tumor suppressor genes. Although most of the tumor-related miRNAs are down-regulated in cancer tissues as compared with their normal counterparts, *miR-21* is one of the few miRNAs that are consistently up-regulated in malignancies of various tissue origins (21), including the breast (22), prostate (23), colon (24), lung (25), stomach (26), liver (27), tongue (18), and brain (28) etc. *miR-21* is significantly up-regulated in a subgroup of breast cancers, and high *miR-21* expression has been associated with unfavorable pathological and molecular features of the disease, including advanced tumor stage, high tumor grade, lymph node metastasis, negative hormone receptor status, and poor patient survival (29). In addition, *miR-21* has been shown to promote oncogenesis (30) and progression (31) of breast cancers *in vitro* and *in vivo*. Intriguingly, the hypothesis of *miR-21* as a central onco-miR in tumor formation is further strengthened by a recent finding demonstrating “onco-miR addition,” in which mice with conditionally expressing *miR-21* develop a pre-B malignant lymphoid-like phenotype, and inhibiting *miR-21* alone induces tumor regression completely (32). Here, our study shows that overexpression of *miR-21* is sufficient to induce trastuzumab resistance in *HER2*⁺ expressing breast cancers *in vitro* and *in vivo*, whereas targeting the onco-miR alone is capable of restoring trastuzumab sensitivity in breast cancer cells with acquired resistance. Likewise, *miR-21* overexpression also contributes to resistance of malignant tumors, including breast cancers, to chemotherapeutic agents, such as paclitaxel (33), docetaxel (34), and gemcitabine (35) etc. Therefore, *miR-21* is not only crucial in the oncogenesis and development of breast cancer, but is also a key factor in the development of resistance to anti-cancer therapies.

In our present study, *miR-21* expression is dramatically increased when *HER2* overexpressing breast cancer cells are induced to acquire trastuzumab resistance by long-term exposure to the antibody in culture. This is inconsistent with the increased homodimerization of EGFR or heterodimerization of EGFR/*HER3* and the elevated levels of phosphorylated AKT after continuous culture of previously sensitive cells in trastuzumab-containing medium, which are suggested as potential molecular mechanisms for acquired trastuzumab resistance (36–37). Because the *miR-21* promoter contains highly conserved regions with consensus binding sites for several transcription factors, including STAT3 (38, 39), AP-1 (40), and factors of the forkhead family (FOXO), such as FOXO3a (41), transcription of primary *miR-21* (*pri-miR-21*) can be regulated via various pathways. Among them, activation of the RAS/MEK/MAPK pathway due to increased homodimerization of EGFR or heterodimerization of EGFR/*HER3* potentially may lead to enhanced transcription of *pri-miR-21* via AP-1 (42, 43). Additionally, phosphorylated activation of AKT may also up-regulate *miR-21* expression by relieving the repression of FOXO transcription factors on its promoter activity (41). On the other hand, *miR-21* may result in EGFR overexpression and homodimerization or heterodimerization with other EGF receptors (44). Likewise, our data demonstrate that *miR-21*

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contributes to sustain AKT phosphorylation in the presence of trastuzumab in the resistant cells. These may feed back to maintain *miR-21* up-regulation in trastuzumab-resistant breast cancer cells. In these scenarios, *miR-21*-mediated trastuzumab resistance is in concert with other resistant mechanisms, and may form positive feed-forward loops with other pathways to exert the resistant effect. The role of *miR-21* in acquired trastuzumab resistance can be reflected by our findings that blocking the action of *miR-21* alone with ASO is sufficient to restore AKT inhibition by trastuzumab in the resistant cells, and is capable of sensitizing the resistant cells to trastuzumab *in vitro* and *in vivo*.

The implications of the above findings spread past acquired trastuzumab resistance, as overexpression of *miR-21* was observed in breast cancers that responded poorly to neoadjuvant trastuzumab therapy. Indeed, a subgroup of breast cancers were reported to express a high level of *miR-21* and stimulation of *HER2/NEU* signaling may trigger *miR-21* up-regulation (45). Moreover, enforced *miR-21* expression in the sensitive breast cancer cells by transfecting its mimics confers trastuzumab resistance in these cells. Therefore, *miR-21* up-regulation is not only crucial to acquired trastuzumab resistance, but may also play a critical role in the inherent primary resistance.

It is well established that miRNAs function via repression of target gene translation, and *miR-21* has a variety of mRNA targets including *PTEN* (20), *CDC25A* (24), *tropomyosin* α_1 (46), *tissue inhibitor metalloproteinase 3* (47), *PDCD4* (48), *RHOB* (31), and *Maspin* (49) validated in different cancer cell types. Evidence suggests that *miR-21* exhibits distinct a biological function dependent on the target genes it silences in a given cancer cell type. Silence of *PTEN* and *PDCD4* results in enhanced proliferation and reduced apoptosis (20, 48), whereas *CDC25A* inhibition is related to cell cycle arrest (24), and tissue inhibitor metalloproteinases, *RHOB*, and *Maspin* are *miR-21* targets for promoting invasion and metastasis (31, 47, 49). Among the *miR-21* targets, *PTEN* deficiency is related to trastuzumab resistance in patients with *HER2*-overexpressing metastatic breast cancer (7). Apart from gene mutation, loss of heterozygosity at the gene locus and epigenetic down-modulation, miRNA dysregulation is also an important causal factor leading to loss of *PTEN* expression and function. Our results further validate that *PTEN* reduction due to elevating *miR-21* is responsible for trastuzumab resistance in *HER2* expressing breast cancer cells. *PTEN* is a tumor suppressing dual phosphatase that antagonizes the function of phosphatidylinositol 3-kinase (PI3K) and negatively regulates AKT activities, and *PTEN* phosphorylation is a crucial mechanism mediating the anti-tumor effect of trastuzumab by reducing and inhibiting the ErbB2 receptor-bound SRC (7). Here we show that rescuing *PTEN* expression in the trastuzumab resistant lines with a *miR-21* ASO or a *PTEN* rescuing vector with the *miR-21* targeting sequence deleted at its 3' UTR restores *PTEN* phosphorylation upon trastuzumab treatment, which ultimately leads to AKT inhibition and growth arrest induction. Moreover, applying the *PTEN* rescuing vector also abolishes trastuzumab resistance induced by *miR-21* mimics. In light of this, blocking the action of *miR-21* with ASO implies tremendous therapeutic value in combination with trastuzumab. Because enforced

PTEN expression or direct inhibition of the AKT signaling pathway is not only technically challenging, but may interfere with normal cellular functions, specifically targeting the up-regulated oncogenic *miR-21* in cancer cells may turn out to be a more feasible therapeutic modality. However, this will require determining how to systemically deliver the ASO into *HER2* expressing cancer cells *in vivo* and selecting delivering vectors with a targeting moiety against the *HER2* receptor, such as a *HER2* antibody fragment fused to protamine (50), may facilitate *in vivo* delivery of *miR-21* ASO for therapeutic purposes.

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